The University of Chicago

THE ANTIGENIC RELATIONSHIP BETWEEN HORSE ANTIBODIES AND THE PROTEINS OF NORMAL HORSE SERUM

A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

1941

By

GEORGE G. WRIGHT, JR.

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THE ANTIGENIC RELATIONSHIP BETWEEN HORSE ANTIBODIES AND THE PROTEINS OF NORMAL HORSE SERUM*

GEORGE G. WRIGHT, JR.†

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The fact that antibodies have specificities as antigens as well as specificities as antibodies has been recognized for many years. Bordet[1] used the term "specificity of origin" to designate this antigenic specificity of antibodies. The results of his experiments show clearly that the antigenic specificity of antibody is the same as the specificity of a substance present in normal serum of the animal. Thus the lysis of sensitized red blood cells by complement was prevented if the sensitized cells were first exposed to the action of the serum of an animal immunized with the normal serum of the animal species supplying the sensitizer. Kraus and Eisenberg[2] had been unable to demonstrate precipitation of antibody from immune serum by precipitating serums prepared against either immune or normal serum of the same species. Hamburger and Dehnes,[3] however, found that when rabbit antihorse serum was added to horse tetanus antitoxin precipitation occurred, and the antitoxin activity was removed from the solution and could be recognized in the precipitate. This precipitation of antibody by a precipitating serum was confirmed by Landsteiner and Prasek[4] with agglutinins, and by Eisler[5] with tetanus antitoxin. Smith and Marrack[6] found that when concentrated horse diphtheria antitoxin was mixed with rabbit antihorse serum in the optimum flocculating proportions of Dean and Webb[7] that the antitoxin was precipitated, and the precipitate would combine with toxin.

There is good evidence that this antigenic specificity of antibody does not involve the part of the antibody molecule which reacts with the antigen. Thus rabbit antipneumococcus antibodies should show no antigenic relationship to horse antipneumococcus antibodies. This conclusion was arrived at by Bordet[1] and has been confirmed by subsequent workers, most recently by Treffers and Heidelberger.[8] Eagle[9] found that when antitoxin was completely saturated with toxin it still precipitated with antihorse serum, and concluded that the combining sites of the antitoxin for the two reagents were different.

The better understanding of the precipitin reaction which followed the work of Dean and Webb[7] yielded a possible explanation for failures to precipitate antibody with precipitating serums. Marrack[10] states, "It is probable that the failures to demonstrate the removal of antibodies from sera by precipitins is due to the use of amounts of precipitin unsuitable for maximum precipitation."

In the course of studies of another na-

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† Mr. and Mrs. Frank G. Logan Research Fellow.

1. Ann. Inst. Pasteur **18:** 593, 1904.
2. Zentralbl. f. bakt. **31:** 208, 1902.
3. Wien. klin. Wchnschr. **17:** 457, 1904.
4. Ztschr. f. Immunitätsforsch. u. exper. Therap. **10:** 68, 1911.
5. Zentralbl. f. Bakt. **84:** 46. 1920.
6. Brit. J. Exper. Path. **11:** 494, 1930.
7. J. Path. & Bact. **29:** 473, 1926.
8. J. Exper. Med. **73:** 293, 1941.
9. J. Immunol. **30:** 339, 1936.
10. The Chemistry of Antigens and Antibodies, Medical Research Council, Special Report Series, no. 230, London, His Majesty's Stationery Office, 1938.

ture the author attempted to precipitate antipneumococcus antibodies from horse serum with rabbit antihorse serum. When the horse serum and the rabbit antiserum were mixed in optimum proportions the antipneumococcus antibody in most cases remained in the supernatant, although the rabbit serums used had high optimum proportions titers. The present study was undertaken in an attempt to determine the conditions for the precipitation of antibody by precipitating serums, and also to throw further light on the general problem of the antigenic specificity of antibody.

MATERIALS

For the preparation of antihorse serum rabbits were immunized by intravenous and intraperitoneal injections of normal horse serum. Antibody titers were determined by the optimum proportions method of Dean and Webb[7] in which concentration of antiserum remained constant. Rabbits 1, 2, 3, 4 and 5 received unpreserved horse serum, and the others received serum preserved with 0.5% phenol. It was important that the serums have high titers, and the immunization was continued, with occasional rest periods, until this condition was fulfilled. Several months were required, the animals receiving at least 20 injections of 5 cc. each. No attempt has been made in the present work to study the effect of different methods of immunization. When high titers were obtained the animals were bled, and the serum preserved with merthiolate and stored in the refrigerator. The titers of the serums did not change appreciably during the course of the experiments. The antipneumococcus horse serum* was an untreated and unpreserved bivalent serum against types I and II. Only the type I antibodies were considered in the present experiments; the agglutinin tier against type I pneumococcus was 1:256. The horse diphtheria antitoxin* was also untreated and unpreserved, and contained about 1200 units/cc. The L^+ dose of the diphtheria toxin* was 0.19 cc. Polysaccharide of type I pneumococcus was prepared by the method of Heidelberger, Kendall, and Scherp.[11] Rabbit antipneumococcus serum was prepared by the immunization of rabbits with a heat-killed virulent culture of type I pneumococcus.

* Obtained from Parke, Davis and Co. through the courtesy of Dr. L. T. Clark.

11. J. Exper. Med. **64:** 559, 1936.

EXPERIMENTAL

Precipitation of Horse Antibodies by Rabbit Antihorse Serum

Antipneumococcus horse serum.— Constant-antiserum optimum proportions ratios of the rabbit antihorse serums with the horse antipneumococcus serum were first determined. The method used was essentially the same as that described by Taylor,[12] which consisted of adding a constant volume of antiserum to a series of dilutions of antigen and observing in which tube or tubes flocculation first occurred. The ratios were first determined roughly, using a large range of serial dilutions of the horse antipneumococcus serum; and then fine tests were set up in which the antigen dilutions were closely spaced in order to make a more accurate determination. Several of the serums showed two or more zones of optimum precipitation, which is evidence of the action of more than one antigen-antibody system. The results of experiments with all the rabbit antiserums are given in table 1, column 1. The ratios are recorded as the amount of horse serum to the amount of rabbit antihorse serum which gave optimum precipitation. That is, if 1 cc. of horse serum diluted 1 to 100 gave optimum flocculation in the presence of 1 cc. of rabbit antihorse serum diluted 1 to 10 the ratio would be 1:10.

The first part of the problem was to ascertain whether antipneumococcus antibodies were precipitated from the optimum proportions mixtures of horse antipneumococcus serum and rabbit antihorse serum. The serums were mixed and placed in the refrigerator for 1 to 2 days. This is the method which was found by Duncan[13] to give the most

12. J. Hyg. **33:** 12, 1933.

13. Brit. J. Exper. Path. **13:** 489, 1932.

TABLE 1.—*Results of Antibody Precipitation and Zone Identification Experiments with Rabbit Antihorse Serums*

Rabbit Antihorse Serum	1 Ratios of Optimum Zones with Horse Antipneumococcus Serum	2 Antipneumococcus Antibody Precipitation in Optimum Mixtures of Rabbit Antihorse and Horse Antipneumococcus Serum	3 Ratios of Optimum Zones with Horse Diphtheria Antitoxin	4 Antitoxin Precipitation in Optimum Mixtures of Rabbit Antihorse and Horse Diphtheria Antitoxin	5 Ratios of Optimum Zones with Albumin from Horse Antipneumococcus Serum	6 Ratios of Optimum Zones with Total Globulin from Horse Antipneumococcus Serum	7 Ratios of Optimum Zones with Water Soluble Globulin from Horse Antipneumococcus Serum	8 Ratios of Optimum Zones with Horse Antitoxin Due to Gamma Globulin and Its Antibody
1	.. 1:12	.. +	1:1.5 1:32	0 +	.. 1:16	.. 1:12	.. 1:12	.. 1:32
2	1:1.5 1:16 1:88	0 0 *	1:1.25 1:7.5 1:300	0 0 +	.. 1:12 ..	1:2 .. 1:64	1:1	.. .. 1:300
3	1:2.5 1:32 ..	0 0 ..	1:2.5 .. 1:100	0 .. +	.. None ..	1:3 1:24 ..	1:2 1:16 ..	.. .. 1:100
4	1:3 1:25 ..	0 0 ..	1:2 .. 1:166	0 .. +	.. 1:16 ..	1:4	1:2	.. .. 1:166
5	.. 1:20	.. +	1:1.25 1:45	0 +	.. 1:16	.. 1:16	.. 1:12	.. 1:45
6	1:7	0	1:4.5	0	None	1:8	1:6	None
7	1:8 ..	0 ..	1:7.5 1:83	0 +	None ..	1:8 ..	1:8 ..	.. 1:83
8	1:3	0	1:4	0	None	1:4	1:3	None
9	1:4 1:100	0 ..	1:3 1:125	0 +	.. 1:64	1:4 1:48	1:4 1:32	.. 1:125

* This ratio probably + (see text).

complete precipitation. Freezing and thawing the mixture, a method used by Smith and Marrack,[6] seemed to give no more complete precipitation. Some difficulty was encountered in devising a satisfactory quantitative method for determining antipneumococcus antibody in the supernatant of such a precipitation. A quantitative method was essential, since in no case were the antibodies completely precipitated, and it is clear that a simple qualitative test for antibody would give erroneous results. Agglutination titrations with pneumococci were first tried, but proved unsatisfactory. In some cases the titer of the supernatant was higher than that of the control series in which the rabbit antihorse serum was replaced by normal rabbit serum. This result was reported by Hyde and Parsons[14] in their study on antiagglutinins to red blood cells. It was found, however, that the supernatant from an optimum mixture of normal horse serum and rabbit antihorse serum would agglutinate a pneumococcus suspension nonspecifically, although neither serum would do so independently. The quantitative antibody nitrogen method (Heidelberger, Sia and Kendall[15]) also proved unsatisfactory, for in preliminary trials more antibody nitrogen was precipitated from some of the supernatants than from the control mixtures in which normal serum had replaced the rabbit antihorse serum. Presumably the polysaccharide antibody complex carried down antihorse antibodies which were

14. Am. J. Hyg. **5:** 245, 1925.

15. J. Exper. Med. **52:** 477, 1930.

probably present as a soluble antigen-antibody complex and were precipitated by the addition of polysaccharide. The method adopted for the present studies was the determination of the combining equivalent of the supernatant for polysaccharide, and is a modification of the method proposed by Felton and Stahl.[16]

To illustrate the method used with all the serums the experiment with serum 5 will be described. This serum gave an optimum ratio of 1:20 with the horse antipneumococcus serum. To 1 of 2 tubes, each containing 0.25 cc. of horse antipneumococcus serum, was added 5 cc. of rabbit antihorse serum 5. To the other was added 5 cc. of normal rabbit serum as a control. The contents were mixed, and the tubes placed in the refrigerator for 2 days. Heavy flocculation occurred in the first tube. The tubes were centrifuged, and the supernatant was distributed in 0.5 cc. amounts in a series of tubes in which varying amounts of polysaccharide (SSS) had been placed as indicated in table 2. The contents were mixed and left overnight in the refrigerator. The tubes were then centrifuged, and each supernatant transferred to a clean tube. The tubes were incubated at 37 C. for 1 hour, and again left overnight in the refrigerator in order to demonstrate that flocculation was complete. Then 0.2 cc. of a 1 to 2 dilution of rabbit antipneumococcus serum was added to each tube, the contents mixed, incubated as before, left overnight in the refrigerator, and examined for flocculation or turbidity. The results are recorded in table 2. The results show that the supernatant from the original precipitation would combine with appreciably less polysaccharide than the control mixture, and that therefore the antipneumococcus antibodies had been precipitated by the rabbit antihorse serum. Similar experiments were carried out with all the zones of optimum precipitation observed with the rabbit serums, except those ratios with serums 2 and 9 in which the amount of horse serum was so small as to make application of the method for determining antibody in the supernatant difficult or impossible. In each case the supernatant was compared with a control mixture in which the rabbit antihorse serum was replaced by normal rabbit serum. The results are summarized in table 1, column 2. An experiment was considered positive for antipneumococcus antibody precipitation if the combining equivalent of the supernatant was reduced to one-third that of the control or less. In those tests whose results are considered negative no appreciable reduction of the combining equivalent was observed. It will be seen that antipneumococcus antibody was precipitated in only 2 of the optimum ratios tested. Five of the rabbit antihorse serums showed no zones in which antipneumococcus antibody was precipitated.

TABLE 2.—*Comparison of the Combining Equivalent for Pneumococcus Polysaccharide of Supernatant from Mixture of Horse Antipneumococcus Serum and Rabbit Antihorse Serum 5 and Control*

SSS I .05 mg. per cc. (cc.)	Salt Solution (cc.)	Supernatant or Control (cc.)	Rabbit Antipneumococcus I Serum 1:2 (cc.)	Precipitation	
				Supernatant	Control
.45	.05	.50	.20	+++	+
.40	.10	.50	.20	+++	Turbid
.35	.15	.50	.20	+++	0
.30	.20	.50	.20	++	0
.25	.25	.50	.20	++	0
.20	.30	.50	.20	+	0
.15	.35	.50	.20	+	0
.10	.40	.50	.20	±	0
	.50	.50	.20	0	0
.10	.40		.20	++	

Horse diphtheria antitoxin.—Experiments were carried out in a similar manner with horse diphtheria antitoxin. The optimum zones of precipitation were de-

16. Standardization of Antipneumococcus Sera and Concentrates, National Institute of Health Bulletin no. 169, Washington, United States Government Printing Office, 1937.

termined in the same manner as with the antipneumococcus serum, and the results are recorded in table 1, column 3. It will be observed that more optimum zones were obtained with the antitoxin than with the antipneumococcus serum. These optimum zones were tested for antibody precipitation, the antitoxin in the supernatants being determined by the Römer intracutaneous technic in rabbits.[17] Since this method is much more sensitive than the method used for the determination of antipneumococcus antibodies in the previous experiments it was possible to apply it to high dilutions of the antitoxin, and this made it possible to test all the zones for antibody precipitation. Optimum flocculating mixtures were set up as before, the amount of antitoxin varying from 0.1 cc. with the zones containing a large amount of antigen to 0.002 cc. with the zones containing small amounts. The supernatants were assayed for antitoxin by setting up serial dilutions, adding an equal volume of diphtheria toxin diluted 1:60, incubating one hour at 37 C., and injecting 0.1 cc. of each mixture into the skin of a rabbit. Several series could be injected into each rabbit, and a control in which the antihorse serum was replaced by normal rabbit serum was injected into each animal. Controls were also included to show that the rabbit serum was not antitoxic, and that the supernatants without toxin did not produce skin reactions in the rabbit. The results of these experiments were clear-cut and satisfactory. In some cases the antitoxin concentration in the supernatant was the same as in the control in which normal rabbit serum replaced the antihorse serum; in others the antitoxin concentration in the supernatant was $\frac{1}{8}$ that of the control or less. These results are shown in table 1, column 4.

17. Jensen, C., Acta path. et microbiol. Scandinav. 1933, supp. 14.

It has already been noted that more zones were observed with the antitoxin than with the antipneumococcus serum. On the assumption that these zones must have been overlooked in the experiments with the antipneumococcus serum, further tests were set up to attempt to detect them. The antigen dilutions were spaced more closely, and the tubes very carefully observed, but no further zones could be detected.

Fractionation of Serum Proteins and Identification of Zones

The foregoing results indicated that in some optimum zones antibodies were precipitated from the horse serum and in others they were not. Goldsworthy and Rudd[18] and Taylor and Adair[19] have shown that the multiple zones obtained in optimum proportions titrations are due to the independent action of different antigens and their antibodies. It seemed of interest, therefore, to determine the antigens responsible for the multiple zones obtained with the rabbit antihorse serums, and attempt to correlate the results with those obtained in the antibody precipitation experiments.

Accordingly, antipneumococcus horse serum was first fractionated into albumin and globulin by half saturation with ammonium sulphate. The precipitated globulin was dissolved in water, and then it and the albumin were dialyzed to remove ammonium sulphate. The fractions were undoubtedly impure, since the precipitation with ammonium sulphate was carried out only once, but it seemed less important to obtain pure fractions than to avoid excessive manipulation of the protein, so that the ratios obtained with the fractions could be compared with those of the whole serum. The fractions were made up to a definite volume so that the concentration in

18. J. Path. & Bact. **40:** 169, 1935.

19. J. Hyg. **35:** 169, 1935.

terms of the original serum was known, and serial dilution optimum proportions titrations were set up with the rabbit antihorse serums. The ratios of the optimum zones obtained with these fractions are recorded in table 1, columns 5 and 6; and are expressed in terms of the original horse serum. It will be noted that each of the zones corresponds in general with a zone obtained with the whole antipneumococcus serum, and all the zones observed with the antipneumococcus serum were observed with the fractions. With rabbit serum 133, for example, the zones at ratios of 1:2 and 1:64 with the globulin fraction correspond with the 1:1.5 and 1:88 ratios obtained with the whole antipneumococcus serum, and the zone at 1:16 with the albumin fraction corresponds with the zone at 1:12 with the whole serum. Since only serial dilutions of the antigens were used in the titrations with the fractions these correlations are as good as could be expected.

Five of the 9 rabbit antihorse serums showed zones with the albumin fraction. No attempt has been made to determine whether these zones with the albumin fraction are due to contamination of the fraction with seroglycoid or other minor components, as suggested by Hewitt.[20] In some cases multiple zones were obtained with the globulin, and further fractionation of this material was undertaken.

The globulin was separated into water soluble and water insoluble fractions by dialysis first against tap water and then distilled water. The precipitate did not all redissolve in salt solution and the solution did not give optimum proportions ratios that correlated with ratios with the whole serum. Presumably this was due to denaturation during the dialysis. However, the supernatant from the dialysis, containing the water soluble globulin, gave satisfactory tests, and the results are recorded in table 1, column 7, The significance of the 2 zones obtained with rabbit serums 3 and 9 against water soluble globulin is open to doubt. These 2 zones might be explained by incomplete removal of the water insoluble globulin.

The experiments with serum 1 were especially interesting; this serum had shown 3 well-spaced zones with antipneumococcus serum. As has already been mentioned, the zone at a ratio of 1:16 was due to albumin, those at 1:1.5 and 1:88 were due to globulin. However only the 1:1.5 zone remained when the water insoluble globulin was removed from the total globulin fraction. The ratios 1:1.5 and 1:16 had not precipitated antipneumococcus antibody, and it is unfortunate that the zone at 1:88 could not be tested directly for antibody precipitation, as already mentioned. An experiment was performed in which it was shown that if the horse antipneumococcus serum was absorbed with pneumococci before it was set up in an optimum proportions titration with serum 2 the zone at a ratio of 1:88 did not appear. This would seem to indicate that the zone at 1:88 was an antibody-precipitating zone, and the fact that it was demonstrated with total globulin but not with water soluble globulin is significant.

Many of the zones with the whole serum were complex; that is, they were composed of superimposed zones due to 2 or more antigens in the horse serum. Thus the zone at a ratio of 1:20 with rabbit serum 5 was shown to be composed of at least 2 independent zones due to albumin and water soluble globulin. It would appear that antibodies against albumin and water soluble globulin do not precipitate antibody; serums 1 and 5 which did precipitate anti-

20. Biochem. J. **31**: 1047, 1937.

body in zones due to albumin and water soluble globulin may have also had a zone due to a water insoluble globulin component superimposed. In order to definitely settle which antigen-antibody system is concerned in antibody precipitation however, it was essential to obtain the antigen which was responsible for zones in which antibody was precipitated and experiment with it directly.

The difficulties and uncertainties of the fractionation of serum proteins by chemical means are well known and it was therefore decided to continue the work with fractions separated by the electrophoretic method of Tiselius.[21] Tiselius found that normal serum may be separated by electrophoretic methods into albumin and 3 globulin fractions which he called alpha, beta, and gamma. Tiselius and Kabat[22] have shown that horse antipneumococcus antibodies migrate as a new globulin component, not present in normal serum, between the beta and gamma fractions. Pappenheimer, Lundgren and Williams[23] have reported similar results with horse diphtheria antitoxin. Van der Scheer, Wyckoff, and Clarke[24] found that antipneumococcus antibodies were associated with the gamma globulin, but confirmed the development of a new fraction in the serum of horses immunized with diphtheria toxin. It seems clear, in any event that antipneumococcus and diphtheria antitoxin antibodies are associated with the slow moving components of horse serum. It seemed reasonable to suppose that antibodies against the normal gamma globulin of horse serum were those responsible for the precipitation of antipneumococcus antibodies and antitoxin antibodies from the horse serum by the rabbit antihorse serums. Studies on the relationship of the gamma globulin to antibody precipitating zones were therefore undertaken.

The apparatus and methods of electrophoresis have been described by Tiselius[21] and others. Normal unpreserved horse serum was diluted with an equal volume of 0.02 molar phosphate buffer at pH 7.6, containing 0.07 molar sodium chloride, dialyzed against more of the buffer and submitted to electrophoresis in a medium capacity separation cell. Migration of the boundaries was followed by visual observation of the schlieren bands, and the compensation movement adjusted at intervals. After twenty hours or more the beta and gamma boundaries were well separated, and the gamma fraction half filled the upper chamber of the cell. The contents of this chamber from two separations were used in the following experiments.

Serial dilution optimum proportions ratios were first determined with the normal gamma globulin and the rabbit antihorse serums used above. Serums 6 and 8 which showed no antibody precipitating zones gave no appreciable precipitation with gamma globulin. The rabbit antihorse serums were then absorbed with optimum amounts of the gamma globulin preparations, and the supernatants set up in optimum proportions titrations with the horse antitoxin. The antitoxin was used instead of the antipneumococcus serum, as in the previous experiments, because more zones were obtained with it, and all the zones had been tested for antibody-precipitation. The antibody-precipitating zones of serums 2, 3, 4, and 7 were removed when the serum was absorbed with gamma globulin, although the other zones with these serums were not changed. Absorption of the antibody-precipitating zones of serums 1, 5, and 9 could not be expected by this procedure, since as has already been pointed out

21. Biochem. J. **31:** 1464, 1937.
22. J. Exper. Med. **69:** 119, 1939.
23. J. Exper. Med. **71:** 247, 1940.
24. J. Immunol. **39:** 65, 1940.

these zones are complex and due to more than one antigen. However, it is clear that the addition of gamma globulin to the horse antitoxin should shift the ratio of zones caused by gamma globulin and its antibody, and not affect the others. By this technic, it was shown that the complex antibody-precipitating zones of serums 1, 5, and 9 contained a zone due to gamma globulin. In addition, the results of the absorption experiments with the other rabbit serums were confirmed by this type of experiment. The ratios of the zones of each rabbit antihorse serum with horse antitoxin which are due to gamma globulin and its antibody are given in table 1, column 8. Studies on the antigenic specificity of the other electrophoretic fractions are in progress.

DISCUSSION

These results indicate that qualifications must be applied to the statement that antibody as an antigenic protein is precipitated when an optimum flocculating amount of antiserum is added. It would appear that when a rabbit is injected with normal horse serum antibodies of several specificities are developed, and that only those reactive with the gamma globulin fraction of the serum are active in precipitating antibodies. Many of the rabbit antihorse serums showed multiple zones of optimum precipitation with horse serums, and with each rabbit serum not more than one of the zones was found to precipitate antibody from the horse serum. Two of the rabbit serums studied would not precipitate antibody from horse serum at all, and these serums gave no reaction with horse gamma globulin. Considerable differences were observed with each rabbit serum between the zones obtained with antipneumococcus and antitoxic horse serums. Presumably this is due to different proprotions of protein components in the 2 horse serums. When antibody-precipitating zones were observed with both horse serums, they occurred at different ratios, and the zones with the antipneumococcus serum occurred uniformly in higher concentrations of horse serum than those with the antitoxin.

It is interesting that more of the rabbit antiserums showed antibody-precipitating zones with the antitoxin than with the antipneumococcus serum. It is doubtful that this indicated any considerable difference in the antigenic specificity of the 2 antibodies. It seems more likely that the explanation lies in the fact that the gamma globulin zones in which horse antibody is precipitated occurred in a higher concentration of horse serum with the antipneumococcus serum, and the ratios were too close to those of the other serum components for them to be resolved. This hypothesis is supported by the fact that the gamma globulin zones, where they occurred alone, were appreciably slower to flocculate than those against the other serum components. Presumably they could easily be obscured by the more rapidly flocculating zones and although antibodies to gamma globulin were present in the rabbit antiserum no zone due to them would be detected. Even though the inapparent gamma globulin zone was reasonably near the visible zone, mixtures in the proportion of the visible zone would not precipitate horse antibody, since the gamma globulin system would be in a region of antigen excess, and precipitation would be largely inhibited. Had the method for determining antibody in the supernatants been applicable to mixtures containing small amounts of horse serum it would have been of interest to determine whether antipneumococcus antibodies would be precipitated when the serums were mixed in proportions containing more rabbit antiserum than the apparent optimum ratio.

Since the amount of each sample of rabbit antihorse serum was somewhat limited it has not been possible to extend the experiments to a number of samples of each type of horse serum. This is of course necessary before it can be determined whether the differences between the antitoxic and antipneumococcic serums are constant properties of these types of serums, or only properties of the particular serums studied. Although the present work does not exclude the possibility of quantitative antigenic differences between antitoxin and antipneumococcic antibodies and normal gamma globulin, it appears that they are certainly qualitatively related.

In view of the results of the present work one is led to speculate why most of the previous workers in this subject seem to have had so little difficulty in precipitating antibodies by precipitating serums. In this connection it may be significant that most of these workers have used commercially concentrated horse serums in their studies, whereas in the present experiments native unpreserved horse serums have been used. It it likely that the use of concentrated preparations would avoid the appearance of some of the zones which have been shown in the present work not to precipitate antibody. But the possibility exists that the antigenic specificity of the antibody might be altered by concentration procedures.

CONCLUSIONS

The present investigation would indicate that the antigenic nature of horse antibody protein is essentially the same as horse normal gamma globulin. The precipitation of horse antibodies from unconcentrated horse serums by optimal flocculating amounts of a rabbit antihorse serum depends not only on whether the rabbit has developed antibodies against gamma globulin, a tendency which seems to vary with different rabbits, but also on more complex factors. If antibodies against gamma globulin are present in a rabbit antihorse serum which shows multiple zones of optimum flocculation with horse serum, only in the zone due to gamma globulin and its antibody will horse antibody be precipitated. If the concentrations of the antigenic proteins in the horse serum and their antibodies in the rabbit serum are such that the slow-flocculating gamma globulin zone is obscured by the other zones no antibody-precipitating zone may be found, even if the rabbit se rum contained antibodies to gammglobulin.

The author wishes to acknowledge his indebtedness and to express his appreciation to Dr. Dan H. Campbell, Dr. L. R. Kuhn, and Dr. Lyman Fourt for their advice and encouragement during the course of this work.